Journal of Physiology (1990), **420**, *pp.* 127–148
With 11 *figures*
Printed in Great Britain

MUSCARINIC AGONISTS AND ATP INCREASE THE INTRACELLULAR Ca^{2+} CONCENTRATION IN CHICK COCHLEAR HAIR CELLS

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(*Received 22 March 1989*)

SUMMARY

1. Cholinergic muscarinic agonists applied by the pressure puff method increased intracellular Ca^{2+} concentration in Fura-2-loaded hair cells. The Ca^{2+} response outlasted the agonist application.

2. The Ca^{2+} response induced by acetylcholine (ACh) was ACh dose dependent with a K_D of 200 μM. Desensitization was negligible, and almost identical Ca^{2+} responses were observed when two ACh puffs were separated by 150 s. The response was blocked by *d*-tubocurarine (*d*TC). The K_D of *d*TC blocking was 500 μM when 100 μM-ACh induced the Ca^{2+} response.

3. The amplitude of the ACh-induced Ca^{2+} responses were potentiated to 3 times the control by incubation with calcitonin gene-related peptide (CGRP; 0·1–1 μM). CGRP did not affect the resting Ca^{2+} concentration. Glycine (100 μM) potentiated the ACh response to 1·4 times the control, and also increased the resting Ca^{2+} concentration slightly.

4. The ACh-induced Ca^{2+} response was suppressed by atropine. It was induced in Ca^{2+}-free extracellular medium, and in Ca^{2+}-free medium desensitization to a second ACh stimulation was significant. The amplitude of the second Ca^{2+} response was 44 % of the first when two ACh puffs were separated by 117 s in Ca^{2+} free medium.

5. Muscarine and carbamylcholine induced similar Ca^{2+} responses, with K_D values of 130 μM for muscarine and 340 μM for carbamylcholine. Desensitization of Ca^{2+} responses was negligible in both agonists.

6. ATP co-exists with ACh in some presynaptic nerve terminals (Burnstock, 1981). Puff-applied ATP (100 μM) generated a Ca^{2+} response with a rapid rising phase and a following slow phase. In Ca^{2+}-free medium the rapid phase disappeared and only the slow phase was observed. The rapid phase is due to the influx of Ca^{2+} ions and the slow phase is due to a release of Ca^{2+} ions from an intracellular reservoir. Under voltage clamp ATP induced a fast inward current and a following slow outward current.

7. Nicotine, adenosine, glycine, GABA, glutamate and bradykinin did not induce Ca^{2+} responses in the hair cell.

8. ACh induced hyperpolarization of the hair cell membrane under current clamp, most probably by the activation of Ca^{2+} activated K^+ conductance. Therefore, a

cholinergic muscarinic receptor may mediate the inhibitory effects of efferent innervation observed in hair cells.

INTRODUCTION

Efferent fibres make axosomatic contacts with outer hair cells and mainly axodendritic contacts onto afferent nerve fibres in inner hair cells of mammals (reviewed by Spoendlin, 1984). Electrical stimulation of these efferent nerves generates inhibitory effects in hair cells and in most primary afferent nerve fibres (Galambos, 1956; reviewed by Klinke & Galley, 1974). Some afferent fibres also receive excitatory efferent innervation (Highstein & Baker, 1985). Acetylcholine (ACh) has been considered the most likely neurotransmitter candidate at these efferent synapses, because of (1) the presence of choline-acetyl-transferase (Jasser & Guth, 1973; Godfrey, Krzanowski & Matschinsky, 1976) and acetylcholine-esterase in efferent nerve fibres and hair cells (Churchill, Schuknecht & Doran, 1956; Dohlmann, Farkashidy & Salonna, 1958; Iurato, Luciano, Pannese & Reale, 1971; Russell, 1971), (2) the inhibitory effects produced by exogenously applied acetylcholine and its agonists (Russell, 1971), (3) the suppression of the efferent stimulation effects by cholinergic antagonists (Russell, 1971) and (4) the increased ACh content of perilymph after efferent stimulation (reviewed by Guth, Norris & Bobbin, 1976). However, ACh is not yet unequivocally determined to be the neurotransmitter of this efferent synapse (reviewed by Klinke & Galley, 1974).

The inhibitory effects generated by efferent stimulation are long lasting (of the order of 100 ms) and are facilitated by multiple efferent stimulation. In turtle hair cells a small depolarizing response was detected at large negative membrane potentials and preceded the hyperpolarization induced by the efferent nerve stimulation. These characteristics of the efferent-induced inhibitory postsynaptic potentials in the turtle hair cells led Art, Fettiplace & Fuchs (1984) to suggest some contribution of intracellular mechanisms to the generation of the slow hyperpolarization.

The presence of cholinergic muscarinic mechanisms at the efferent synapse has been suggested by several groups (Steinacker & Rojas, 1988; van Megen, Klaassen, Rodrigues de Miranda & Kuijpers, 1988). Cholinergic muscarinic receptors are known to activate a K^+ conductance in sympathetic ganglion cells (reviewed by Horn & Dodd, 1983) and in other central neurones (McCormick & Prince, 1986; Egan & North, 1986), while other cholinergic muscarinic receptors suppress K^+ conductance and cause depolarization of the membrane (reviewed by Krnjević, 1974). Muscarinic activation of K^+ conductances could be due to an increase in the intracellular Ca^{2+} concentration mediated probably by the turnover of phosphatidylinositol (Berridge & Irvine, 1984). In the present study the possible involvement of such cholinergic muscarinic mechanisms in the efferent synapse to hair cells was studied by applying Fura-2 Ca^{2+} indicator dye techniques to the dissociated hair cell preparation of chick cochlea.

METHODS

Preparations and solutions

Hair cells were dissociated from the cochlear organ of chicks. Detailed procedures were the same as previously reported (Ohmori, 1984), with the exception that we have not used proteolytic enzymes. Hair cells were dissociated by trituration in Ca^{2+}, Mg^{2+}-free balanced salt solution, containing DNAse (1 mg/ml, D-5205, Sigma).

Experiments were performed in normal saline containing (mM): NaCl, 155; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 17; buffered to pH 7·4 by 10 mM-K^+-HEPES). $CaCl_2$ and $MgCl_2$ were replaced with NaCl in Ca^{2+}, Mg^{2+}-free medium. The bath solution was continuously circulated (3–5 ml/min) to facilitate washing out of pressure-applied agonists. Clearance speed of the puff-applied agonists was evaluated in a control experiment where a fluorescence from the pressure-ejected dye was cleared from around the pipette within a single measurement cycle (3 s). Dissociated hair cells and hair cell clusters were plated onto a lectin-coated cover-glass which was the floor of the recording chamber (volume 500 μl). The walls of the recording chamber were made of aluminium for better heat conduction, and were coated with Teflon. The chamber was mounted on a pair of Peltier plates (Thermo-module, CP 1·4-71-06L, Netsu Denshi Co. Ltd, Osaka, Japan) to maintain the bath temperature at 33–37 °C during experiments.

Loading hair cells with Fura-2

After trituration, hair cells were incubated with the membrane-permeable dye Fura-2 AM (acetoxymethyl ester form, Molecular Probes Inc., Junction City, OR, USA) at a concentration of 3·3 μM in high-glucose medium containing (mM): glucose, 90; NaCl, 100; $CaCl_2$, 2·5; $MgCl_2$, 1; buffered to pH 7·4 by 10 K^+-HEPES; enriched with BSA, 2 mg/ml, for 30–40 min at 37 °C. Hair cells were then rinsed with normal saline and were incubated for 15–30 min at 33–37 °C.

Calcitonin gene-related peptide (CGRP, 0·1–1 μM) was added to the Ca^{2+}, Mg^{2+}-free medium during trituration and to the high-glucose medium during Fura-2 incubation. CGRP incubation was for about 1 h.

Excitation and observation of Fura-2 fluorescence

Fluorescence of Fura-2-loaded hair cells was observed by essentially the same method described previously (Ohmori, 1988). The following descriptions are limited to new developments and improvements of the method. Fluorescence was observed by an Olympus UVFL × 40, numerical aperture = 0·85, with a dry objective lens attached to an inverted microscope (IMT-2, Olympus, Tokyo, Japan) illuminated by a 75 W xenon short arc lamp (L2194-01, Hamamatsu Photonics, Hamamatsu, Japan).

Fluorescence was excited at wavelength of 340 and 380 nm. By taking ratios of the 340 and 380 nm fluorescence, the intracellular Ca^{2+} concentrations could be estimated (Grynkiewicz, Poenie & Tsien, 1985). The fluorescence ratios were calibrated by using EGTA-Ca^{2+} buffer solutions with Fura-2/5K (penta-potassium salt). The fluorescence ratio was 0·3 in a 4 mM-EGTA solution, and was 5·9 in a 1 mM-Ca^{2+} solution. The average resting fluorescence ratio of the hair cell was 0·94, suggesting an intracellular Ca^{2+} concentration of 150 nM. However, rather than describing the absolute intracellular Ca^{2+} concentration, we have described 340/380 nm fluorescence intensity ratios in this paper. The excitation wavelengths were limited by using interference filters (340 nm, half-width 13 nm, 56% transmission; 380 nm, half-width 10 nm, 75% transmission). The intensity of 380 nm light was reduced to 6% and the 340 nm to 12% by a pair of neutral density filter in order to reduce the fluorescence bleaching and to match the fluorescence intensities excited at these two wavelengths.

Hair cells were illuminated at 340 and 380 nm wavelengths by exchanging two interference filters mounted on a slider (OSP-3/2EX, Olympus, Tokyo, Japan). The time resolution was limited since approximately 150 ms was required for the exchange and settling of the interference filters, but this method was ideal for fluorescence imaging techniques where 340 and 380 nm fluorescence images were frame-integrated into two separate frame memories. Fluorescence ratio measurements were made from up to ten locations at 3 s intervals. Sampling locations were indicated on the fluorescence image by squares corresponding to the place and the size of the measurement area (Figs 1 and 2).

Fluorescence was monitored through a 500 nm interference filter (half-width 20 nm, 88% transmission) and imaging was made by a video-intensified microscope system (AVEC/VIM

system, Hamamatsu Photonics, Hamamatsu, Japan) which was controlled by a host computer (micro VAX-II/GPX, D.E.C., Maynard, MA, USA). Fluorescence images of the whole observation area excited at either wavelength before and after the experiment and fluorescence intensity data measured at 3 s intervals were stored permanently on an optical disc unit (Toshiba, Tokyo, Japan). Fluorescence images were hard-copied by a video printer (Mitsubishi electric, Tokyo, Japan). Unless otherwise noted the data presented in this paper will be in the form of mean ± standard error of the mean (S.E.M.).

Experimental procedures

Agonists were pressure puff-applied to hair cells using glass capillaries of 3–5 μm tip diameter. The tip of the glass capillary was placed about 100–150 μm away from the preparation. Ca^{2+}, Mg^{2+}-free saline was applied to hair cells by a fast microperfusion method similar to the one developed by Krishtal & Pidoplichiko (1980). The tip of the microperfusion system was about 200 μm diameter and was placed about 500 μm away from the preparation and opposite the agonist application pipette. The puff pressure and the microperfusion flow rate were adjusted in order to achieve rapid application of the agonist solution or the bathing solution while avoiding dislocating hair cells under observation. When hair cells were disturbed, fluorescence intensities at both wavelengths changed in parallel in the same direction. While the extent of displacement was small the ratio could be compensated for these movements (see spike-like artifact in traces 2, 3, and 4 at about 160 s in Fig. 10*A*). When large motion artifacts were observed, data were discarded.

Electrical recordings were made from isolated hair cells by applying the whole-cell patch-electrode-recording technique. Detailed procedures were described previously (Ohmori, 1984). The patch electrode was filled with KCl-EGTA-based intracellular medium containing (mM): KCl, 155; EGTA, 5; buffered to pH 7·4 by 10 K^+-HEPES. Agonists were puff-applied to the hair cell either under current clamp or under voltage clamp conditions. The puff was for 1 s and was synchronized by a programmable stimulator (OI-8, Shoshin EM, Okazaki, Japan) to the A/D samplings of membrane current and voltage.

Cholinergic agonists, antagonists and other chemicals

The following substances were used in the experiment: acetylcholine-Cl (A-6625, Sigma), adenosine (A-9251, Sigma), amiloride (A-7410, Sigma), ATP (A-0770, Sigma), atropine (A-0132, Sigma), bradykinin (B-3259, Sigma), carbamylcholine (C-4382, Sigma), calcitonin gene-related peptide (CGRP, Peptide Institute, Osaka, Japan), *d*-tubocurarine (*d*TC; 207–03431, Wako Pure Chemical Industry), GABA (A-2129, Sigma), muscarine (M-7756, Sigma), streptomycin-sulphate (S-6501, Sigma).

RESULTS

Acetylcholine induces increases of intracellular Ca^{2+} concentration

When 100 μM-acetylcholine (ACh) was puff-applied to a solitary hair cell, Fura-2 fluorescence was modified (Fig. 1). The fluorescence excited at the 340 nm wavelength was increased and the fluorescence excited at the 380 nm wavelength was decreased reciprocally after ACh application (Fig. 1*B*). These changes of fluorescence indicate an increase of intracellular Ca^{2+} concentration. The fluorescence ratio calculated from these two measurements at each time interval was increased from the pre-stimulation level of 0·75 to the peak level of 1·05 in about 100 s. The timing of ACh application is indicated by a bar on the abscissa and ACh was applied for 40 s in this example (Fig. 1*C*). The rise in intracellular Ca^{2+} concentration outlasted the period of ACh application. Since Fura-2 fluorescence reflects the Ca^{2+} concentration with relatively high specificity (Grynkiewicz *et al.* 1985), we may describe these ratio changes as changes of intracellular Ca^{2+} concentration hereafter.

Fluorescence was measured at four overlapping locations in this hair cell (Fig. 1*A*). The hair cell surface area covered by each measurement location was not equal and was largest in location 2 and was smallest in location 1 which covered the hair bundle

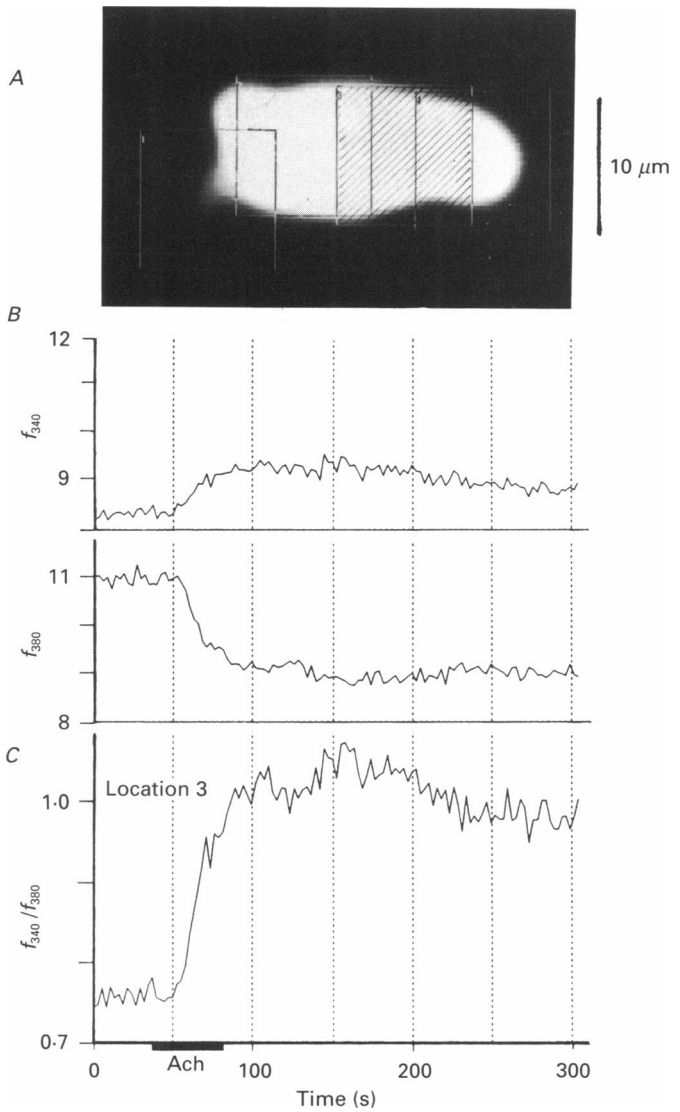

Fig. 1. Fluorescence changes generated by an ACh (100 μM) puff on a Fura-2-loaded hair cell. *A*, Fura-2 fluorescence image of an isolated hair cell. Fluorescence intensity measurements were made from four locations, and their areas are indicated by squares on the hair cell. Location 3 is hatched and its fluorescence changes are indicated in *B*, *B*, fluorescence intensity changes measured by exciting the cell at wavelengths of 340 nm (f_{340}) and 380 nm (f_{380}). The ordinate is in arbitrary units. *C*, the ratio intensity f_{340}/f_{380} at each time interval was calculated from the measurements in *B*. The timing of the 100 μM-ACh puff application is indicated by a bar on the abscissa. The puff pipette was located on the right of the cell and the ACh puff was directed to the basal end of the cell. Note the ratio change outlasted the ACh pressure puff.

and a part of the cuticular plate. The fluorescence and the ratio measured in location 3 was presented in Fig. 1 (hatched area in Fig. 1*A*). The absolute intensity of fluorescence was different depending on the hair cell region covered, but the fluorescence ratio and the time course of the ratio changes were almost the same, and could be superimposed on each other. The time resolution of 3 s of the present measurements may have been too slow to detect possible local changes in intracellular Ca^{2+} concentration.

When fluorescence was measured from a cluster of hair cells, each measurement area was placed in order to cover fluorescence from a single hair cell. As shown in Fig. 2, when 100 μM-ACh was puff-applied, all ten hair cells simultaneously monitored demonstrated increases in intracellular Ca^{2+} concentration. In this experiment, the ACh puff pipette was placed on the right of the cell cluster close to location 7, but the Ca^{2+} response amplitude was not necessarily large closer to the puff pipette and the time course varied from cell to cell. All Ca^{2+} responses outlasted the ACh puff (indicated by a bar on the abscissa).

Possible mechanical stimulation applied to the hair bundle by the pressure puff of ACh might have allowed a small amount of Ca^{2+} influx through the mechano-electrical transduction (MET) channel (Ohmori, 1988). However, the rise of intracellular Ca^{2+} concentration monitored here was not explained by this Ca^{2+} influx through the MET channel alone. (1) The intracellular Ca^{2+} response clearly outlasted the puff stimulation while the MET current induced by puff stimulation of the hair bundle closely followed the time course of the puff (Fig. 2*A*, Ohmori, 1988). (2) Moreover, these Ca^{2+} responses were not always induced by the puff application of the agonist: they were not induced by a puff of nicotine (100 μM and 1 mM), adenosine (100 μM), glutamate (100 μM), glycine (100 μM), CGRP (1 μM), GABA (10 mM), and bradykinin (20 μM). (3) Amiloride at 0·5 mM did not suppress the Ca^{2+} response to 100 μM-ACh (Ca^{2+} response in 0·5 mM-amiloride saline was 63 ± 10% (mean $\pm$ S.E.M., $n = 8$) of the control), while this concentration of amiloride reduced the MET current to 10% of the control (Jørgensen & Ohmori, 1988). Streptomycin at a concentration of 0·1 mM has reduced the Ca^{2+} response to 51 ± 11% ($n = 12$) of the control. This concentration of streptomycin reduces the MET current but is not enough to block it completely (Ohmori, 1985). Both of these MET channel blocking agents to some extent affected the Ca^{2+} response generated by ACh, but the extent of interference was much smaller than their effects on the MET channel.

CGRP potentiates the ACh-induced intracellular Ca^{2+} response

Calcitonin gene-related peptide (CGRP) has been found at the synaptic region of the outer hair cell of rat (Kitajiri, Yamashita, Tohyama, Kumazawa, Takeda, Kawasaki, Matsunaga, Girgis, Hillyard, MacIntyre, Emson, Shiosaka & Tohyama, 1985), and has been further demonstrated in the major fraction of cochlear efferent fibres of the olivocochlear bundle in rat (Takeda, Kitajiri, Girgis, Hillyard, MacIntyre, Emson, Shiosaka, Tohyama & Matsunaga, 1986). CGRP is known to co-exist with ACh in motor nerve terminals (Takami, Kawai, Shiosaka, Lee, Girgis, Hillyard, McIntyre, Emson & Tohyama, 1985) and at the neuromuscular junction it is a motoneurone-derived trophic factor that increases ACh-receptor synthesis (New & Mudge, 1986). The chronic application of CGRP has prevented disuse-induced

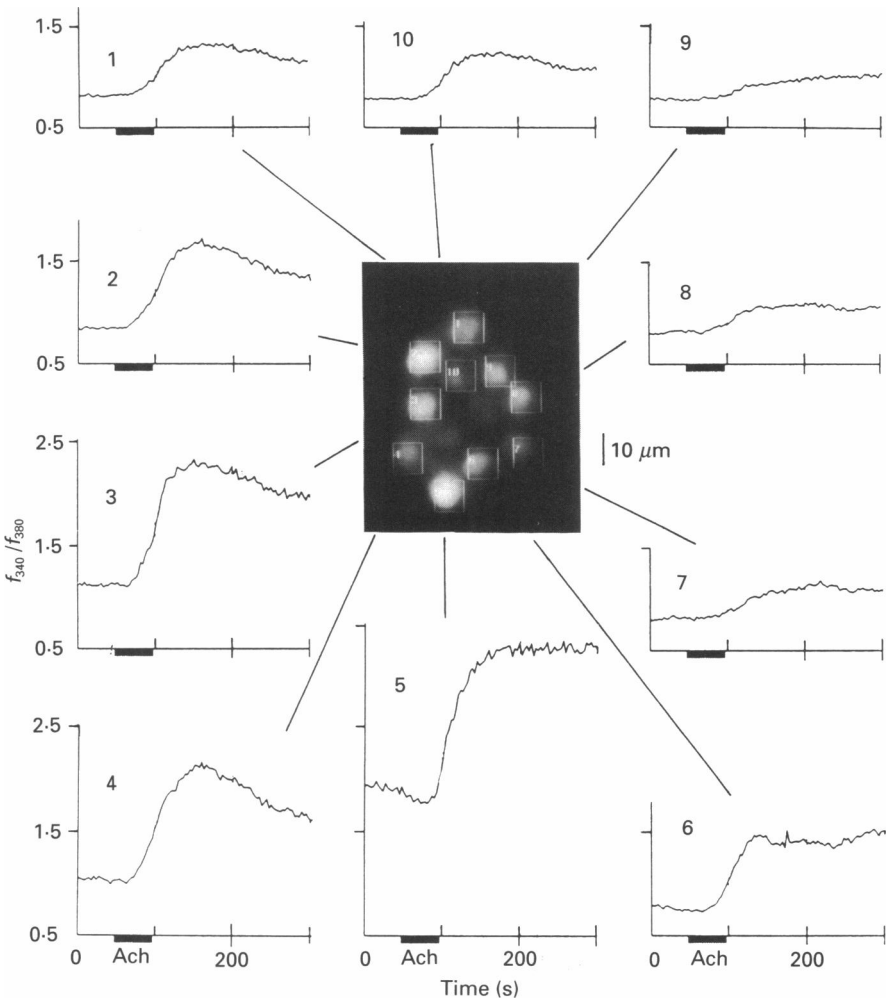

Fig. 2. Fluorescence ratio changes measured simultaneously from ten hair cells. Fluorescences at 340 and 380 nm excitation wavelengths were measured from these ten hair cells presented in the inset picture. Measured cells and corresponding measurement areas are marked by squares. One hundred μM-ACh was puff-applied from the right of the preparation close to location 7 and was directed towards left. The timing of the ACh puff is indicated by a bar on the abscissa of each ratio measurement.

sprouting of motor nerve terminals, and has increased the frequently of the miniature endplate potentials (Tsujimoto & Kuno, 1988). Hair cells were therefore incubated with 0·1–1 μM CGRP for 1 h during the whole course of trituration and Fura-2 incubation, in order to see whether CGRP has modulatory effects on the ACh-induced intracellular Ca^{2+} responses or not.

Puff-applied CGRP at 1 μM did not generate intracellular Ca^{2+} responses, while ACh applied at 100 μM to the same hair cells increased intracellular Ca^{2+} concentration (seventeen hair cells). CGRP (1 μM) puff-applied together with ACh

(100 μM) did not generate larger Ca^{2+} responses than the control 100 μM-ACh puff (thirteen hair cells). Incubation with CGRP did not change the resting cytosolic Ca^{2+} concentration. The mean fluorescence ratio (R_0) was $0{\cdot}92 \pm 0{\cdot}04$ ($n = 91$) when hair cells were not incubated with CGRP and was $0{\cdot}95 \pm 0{\cdot}02$ ($n = 163$) when the cells were

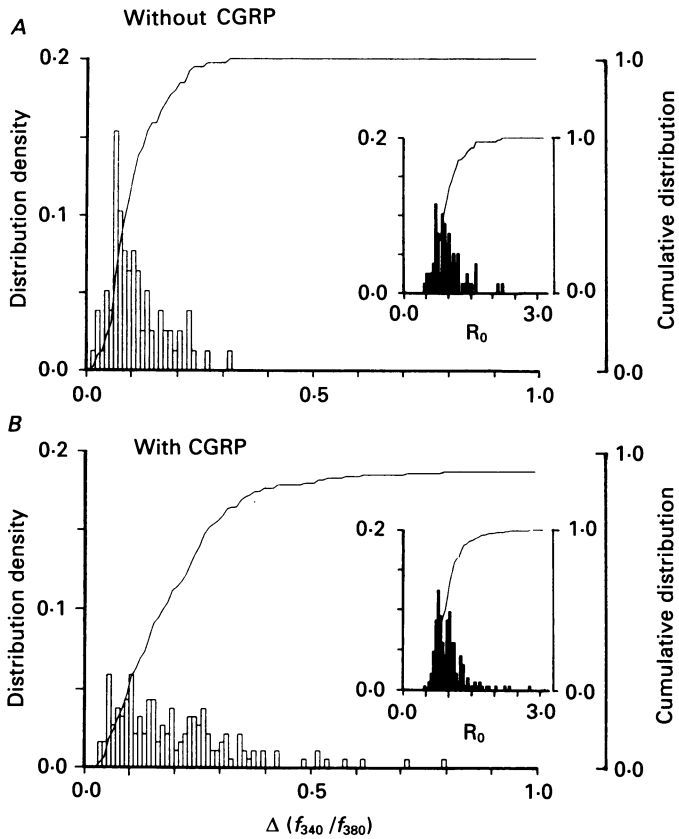

Fig. 3. Calcitonin gene-related peptide (CGRP) potentiates the ACh response. Intracellular Ca^{2+} responses were generated by pressure puff of 100 μM-ACh. *A*, a distribution of ratio changes ($\Delta(f_{340}/f_{380})$) measured from ninety hair cells without CGRP incubation. The ordinate on the left indicates a probability density distribution of the ratio change. The smooth line indicates a cumulative distribution of ratio changes and the ordinate is on the right. The inset histogram and the cumulative distribution illustrate the resting ratio distribution (R_0, mean $\pm$ S.E.M., $0{\cdot}92 \pm 0{\cdot}04$, $n = 91$). *B*, distribution of ratio changes measured from 185 hair cells incubated with 1 μM-CGRP for 1 h. The slope of the cumulative distribution was not as steep as in *A*, which indicates a higher incidence rate of large amplitude ratio changes in CGRP-incubated cells than in cells without CGRP incubation. The inset histogram and the cumulative distribution show resting ratio distribution ($0{\cdot}95 \pm 0{\cdot}02$, $n = 163$).

incubated for 1 h with CGRP (inset in Fig. 3*A* and *B*). However, when response amplitudes were compared, hair cells incubated with CGRP generated larger Ca^{2+} changes than those without incubation (Fig. 3). The ratio change ($\Delta(f_{340}/f_{380})$) induced by 100 μM-ACh puff was $0{\cdot}30 \pm 0{\cdot}03$ ($n = 185$) in cells incubated with CGRP

and $0{\cdot}10 \pm 0{\cdot}01$ ($n = 90$) in control cells without CGRP incubation. When hair cells were incubated with CGRP the incidence of relatively large Ca^{2+} responses was increased. These changes in the Ca^{2+} response amplitude were reflected in a less steep rise of the cumulative distribution of ratio changes in CGRP-incubated hair cells (Fig. 3*B*).

Glycine might potentiate ACh-induced Ca_i^{2+} responses

When glycine (100 μM) was present in the bathing medium for 5–10 min, intracellular Ca^{2+} responses generated by 100 μM-ACh were potentiated to $136 \pm 3\%$ ($n = 35$) of the control. Puff-applied glycine did not generate Ca^{2+} responses nor potentiate the Ca^{2+} responses when applied together with ACh. Hair cells incubated with glycine demonstrated relatively larger resting cytosolic Ca^{2+} concentration than those without incubation. The resting fluorescence ratio was $0{\cdot}85 \pm 0{\cdot}03$ ($n = 14$) without glycine, and was $1{\cdot}07 \pm 0{\cdot}02$ ($n = 24$) when 100 μM-glycine was present in the bathing medium.

ACh dose vs. intracellular Ca^{2+} responses

A relatively high concentration of ACh (100 μM) was puff-applied in these experiments in order to generate intracellular Ca^{2+} responses reliably. Ca^{2+} responses could be generated at much lower concentrations of ACh. The ACh dose dependence of the intracellular Ca^{2+} response was therefore studied. Ca^{2+} responses were measured at various ACh concentrations and the relative response amplitude was calculated against the Ca^{2+} response generated in the same hair cell by a 100 μM-ACh puff. With higher concentrations of ACh a sufficient time interval was allowed between the control and test trial (more than 400 s). As is illustrated in Fig. 4*C*, desensitization to 100 μM-ACh was small, and the amplitude of the second Ca^{2+} response was $90{\cdot}4 \pm 1{\cdot}6\%$ ($n = 87$) of the first Ca^{2+} response when two ACh puffs were separated by 72–223 s (151 ± 43 s, S.D. ($n = 87$)). Figure 4*A* and *B* show a series of ACh responses which are already scaled in amplitude in each pair by making the control Ca^{2+} response induced by 100 μM-ACh a constant. The amplitude of the control Ca^{2+} response was scaled using the 100 μM-ACh in Fig. 4*C* as a reference. Figure 4*A* is a series without CGRP incubation and Fig. 4*B* is with CGRP incubation. Since a series with CGRP incubation was recorded with 50 μM-ACh as the control, the amplitude of these Ca^{2+} responses was plotted (●) in Fig. 4*D* relative to the response to 50 μM under each condition. These two series of ACh responses determined a single dose *vs.* response relationship, with a Hill coefficient of 1 and a K_D of 200 μM. The maximum response amplitude was set at 2·8 times that of 100 μM-ACh in Fig. 4*D* and was indicated by an arrow on the ordinate.

dTC and atropine suppress the ACh-induced Ca^{2+} rise

The inset in Fig. 5 illustrates suppression of the Ca^{2+} response by 1 mM-*d*TC applied together with 100 μM-ACh. *d*TC was puff-applied together with ACh 530 s after the control response was elicited. The amplitude was reduced to 33% of the control by *d*TC. The ACh-induced Ca^{2+} response recovered to the 87% of the control in this cell. ACh responses were suppressed to an average of $32 \pm 3\%$ ($n = 14$) of the

control by 1 mM-*d*TC. The dose–response relationship plotted in Fig. 5 had a K_D of 500 μM, and a Hill coefficient of 1.

When 10 μM-atropine was bath applied, puff application of 100 μM-ACh did not induce Ca^{2+} responses in three out of seven hair cells. The other four cells generated

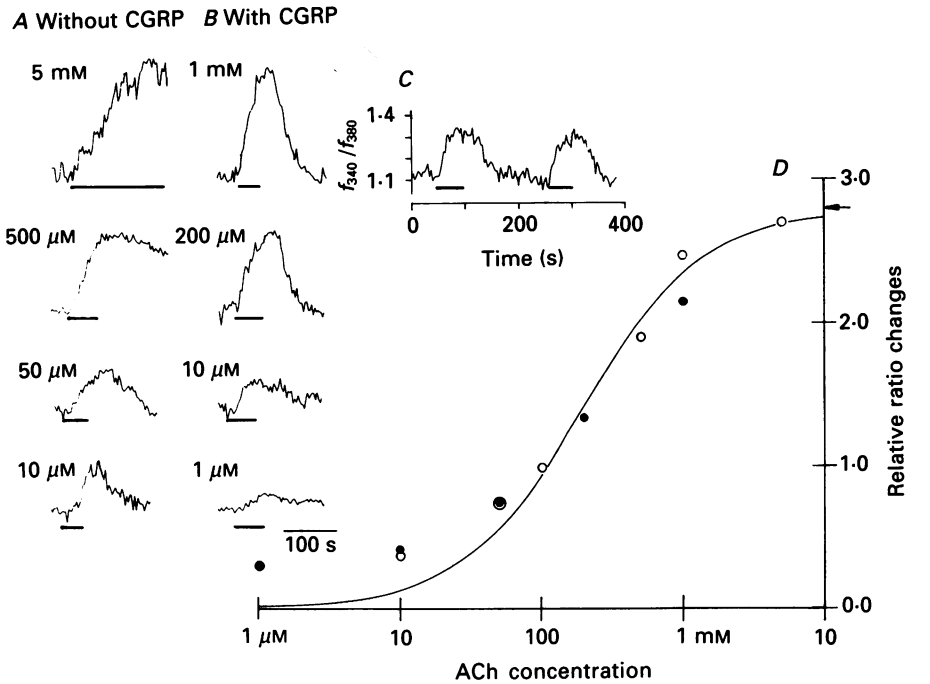

Fig. 4. Dose dependence of ACh-induced intracellular Ca^{2+} changes. Relative amplitudes of Ca^{2+} responses generated by a puff of ACh at various concentrations are shown after scaling using 100 μM-ACh response as a reference. The ACh concentration is indicated on the left and the duration of ACh application is indicated by a bar in each trace. *A*, Ca^{2+} responses in hair cells without CGRP incubation. *B*, Ca^{2+} responses in hair cells after CGRP incubation for 1 h. *C*, two identical ACh applications (100 μM) were separated by 150 s. Note the amplitudes of these two Ca^{2+} responses were the same. *D*, relative amplitudes of Ca^{2+} responses generated by various ACh concentrations plotted on a semilogarithmic co-ordinate. ○, responses in cells without CGRP incubation (10 μM, 0·394±0·04, $n = 13$; 50 μM, 0·748±0·05, $n = 8$; 500 μM, 1·88±0·13, $n = 22$; 1 mM, 2·47±0·33, $n = 15$; 5 mM, 2·70±0·33, $n = 15$); ●, responses from cells after CGRP incubation (1 μM, 0·356±0·02, $n = 15$; 10 μM, 0·435±0·03, $n = 18$; 50 μM, 0·748; 200 μM, 1·33±0·15, $n = 12$; 1 mM, 2·14±0·15, $n = 10$). The continuous line is drawn using the Michaelis–Menten theory with a K_D of 200 μM and a Hill coefficient of 1. The arrow on the ordinate indicates a saturation level of 2·8.

small Ca^{2+} responses which were 44±3% ($n = 4$) of control responses (Fig. 6*A*). When 100 μM-atropine was bath applied it blocked the ACh response in all twelve hair cells studied. These atropine effects were reversible. When 100 μM-atropine was applied only in the puff together with ACh (100 μM), small Ca^{2+} responses were observed in fourteen out of twenty cells studied. Atropine reduced Ca^{2+} responses to 43±18% ($n = 14$) of the control. This suppression by atropine was reversible (Fig. 6*A*). While we have not studied the dose–response relationship in detail, atropine

seems to be at least one order of magnitude more potent than *d*TC. This suggests that a muscarinic cholinergic receptor mechanism mediates the ACh-induced rise of intracellular Ca^{2+} concentration in the hair cell.

ACh induces intracellular Ca^{2+} responses in Ca^{2+}-free extracellular medium

Activation of some type of cholinergic muscarinic receptor is known to facilitate phosphatidylinositol turnover (reviewed by Nathanson, 1987) and generate inositol

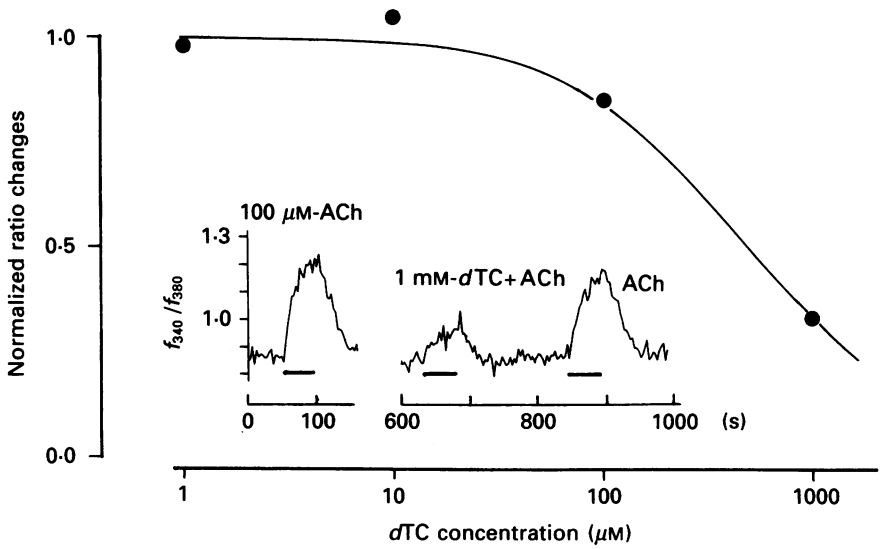

Fig. 5. *d*TC block of the ACh-induced Ca^{2+} response. *d*TC at various concentrations was puff-applied together with 100 μM-ACh to hair cells. The inset illustrates a partial block of 100 μM-ACh responses by 1 mM-*d*TC. The *d*TC effect was reversible. Amplitudes relative to the 100 μM-ACh response are plotted: 1 μM, 0·974±0·08 ($n = 20$); 10 μM, 1·049±0·07 ($n = 10$); 100 μM, 0·844±0·06 ($n = 23$); 1 mM, 0·324±0·03 ($n = 14$). The continuous line was drawn using the Michaelis–Menten theory with a K_D of 500 μM and a Hill coefficient of 1.

trisphosphate (IP_3). IP_3 induces release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). We measured intracellular Ca^{2+} responses in 133 hair cells in Ca^{2+}, Mg^{2+}-free extracellular medium. In these experiments, hair cells were bathed in Ca^{2+}, Mg^{2+}-free extracellular solution for at least 5 min before ACh puff stimulation. Only seventeen cells demonstrated a Ca^{2+} rise in response to the 100 μM-ACh puff. The time courses of these responses were similar to those observed in Ca^{2+}-containing medium, and outlasted the ACh puff but the response amplitude was small (ratio change was 0·034±0·003, $n = 17$). This observation that only small responses were obtained in small proportion of cells (13%) in Ca^{2+}-free medium suggests that Ca^{2+} influx may be the major source of the ACh-induced Ca^{2+} response. Alternatively there may be a small intracellular Ca^{2+} reservoir which depletes quickly in the Ca^{2+}-free extracellular medium. We have therefore adopted a technique of a fast microperfusion in order to rapidly apply Ca^{2+}-free extracellular medium to the surroundings of hair cells.

When Ca^{2+}-free medium was microperfused about the hair cells, the resting fluorescence ratio decreased, indicating a fall in intracellular Ca^{2+} concentration (at 316 s in Fig. 6*B*). ACh (100 μM) in Ca^{2+}-free solution was then puffed against the hair cell, and a rise in intracellular Ca^{2+} concentration was induced (at about 400 s in Fig.

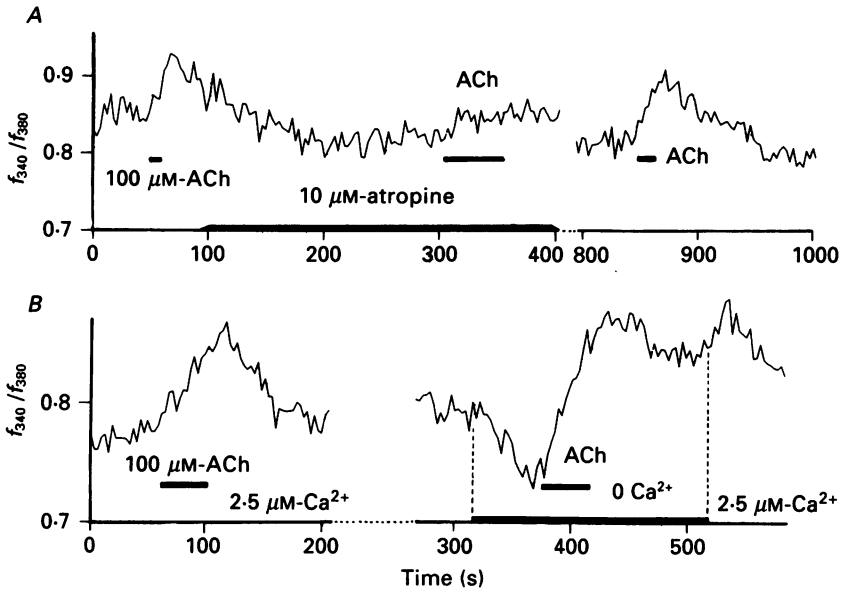

Fig. 6. ACh responses are blocked by atropine and are generated in Ca^{2+}-free extracellular medium. *A*, a bath application of 10 μM-atropine blocked most of the Ca^{2+} response generated by 100 μM-ACh. The application of atropine is indicated by a bar on the abscissa. The blocking effect was reversible. *B*, Ca^{2+} response was generated in Ca^{2+}-free extracellular medium applied rapidly by a microperfusion method. The flow of Ca^{2+}-free medium is indicated by a bar on the abscissa. After application of Ca^{2+}-free medium the intracellular Ca^{2+} concentration is decreased. A flow of 2·5 mM-Ca^{2+} extracellular medium introduced by suspending the microperfusion of Ca^{2+}-free solution induced a transient rise of intracellular Ca^{2+} concentration. These two timings are indicated by two vertical dashed lines.

6*B*). This Ca^{2+} response was similar to the one observed in normal saline, and clearly outlasted the ACh puff. At 516 s microperfusion of Ca^{2+}-free saline was suspended, which permitted 2·5 mM-Ca^{2+} extracellular medium to come in contact with the cell membrane and caused a transient rise in intracellular Ca^{2+} concentration. Under Ca^{2+}-free medium which was quickly introduced by the fast microperfusion method, ratio changes were $0{\cdot}12 \pm 0{\cdot}10$ ($n = 34$) and were measured in thirty-four hair cells out of sixty cells studied (57 %).

When two identical ACh puff stimuli were applied in Ca^{2+}-free medium, the second Ca^{2+} response was $44{\cdot}2 \pm 0{\cdot}2$ % ($n = 18$) of the control when the two stimuli were separated by 117 ± 11 s, S.D. ($n = 18$). This reduction was much greater than that seen in normal saline, where the second Ca^{2+} response was $90{\cdot}4 \pm 1{\cdot}6$ % ($n = 87$) of the first one when separated by 151 ± 43 s, S.D. ($n = 87$). The second Ca^{2+} response in the Ca^{2+}-free medium was therefore 48 % of the second Ca^{2+} response in normal medium. This may indicate a relatively easy depletion of Ca^{2+} ions from the intracellular stores.

Ca^{2+} responses generated by muscarinic agonists

When muscarine and carbamylcholine were puff-applied to the hair cell, Ca^{2+} responses were generated. The time course was similar to the one induced by ACh puff and the response outlasted the period of agonist application. When two

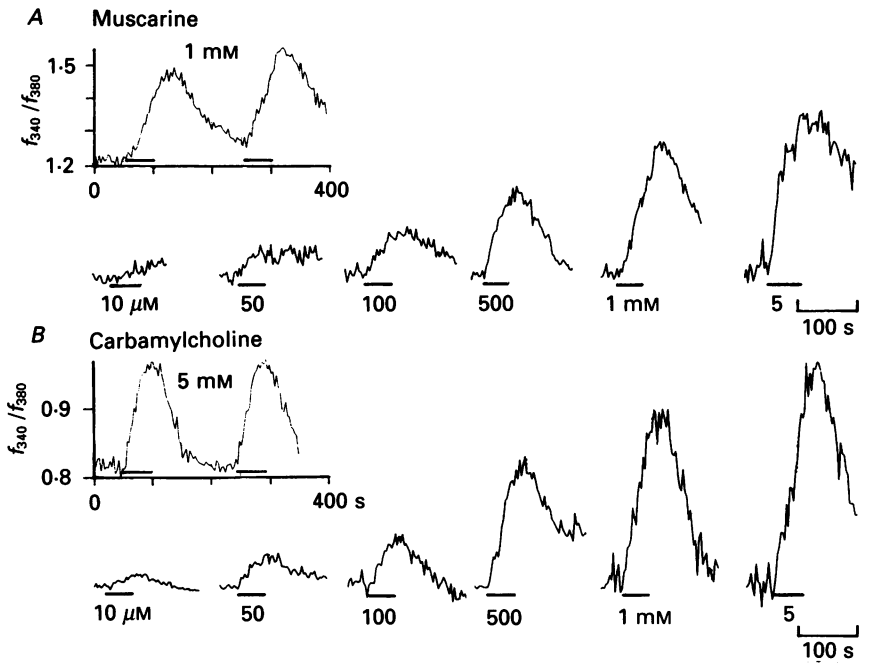

Fig. 7. Relative amplitudes of muscarine- and carbamylcholine-induced Ca^{2+} responses. The concentration of agonist and the period of puff application (bar) is indicated in each trace. *A*, relative amplitude of muscarine-induced Ca^{2+} responses. The inset trace demonstrates Ca^{2+} responses induced by two successive 1 mM-muscarine puffs. *B*, relative amplitudes of carbamylcholine-induced Ca^{2+} responses. The inset trace demonstrates Ca^{2+} responses induced by two successive puffs of 5 mM-carbamylcholine.

muscarine (1 mM) puffs were applied in succession at 150 s intervals the amplitude of the second Ca^{2+} response was $96{\cdot}4 \pm 3{\cdot}1\%$ ($n = 9$) of the control response (Fig. 7*A*). Muscarine at various concentrations was applied (Fig. 7*A*) to determine a dose dependence of muscarine-induced Ca^{2+} responses. The response to 100 μM-muscarine was used as a reference. Relative amplitudes of Ca^{2+} responses are plotted in Fig. 8 (○). In these experiments, 5 mM-muscarine induced a Ca^{2+} response $2{\cdot}51 \pm 0{\cdot}14$ ($n = 25$) times larger than 100 μM-muscarine. Therefore, all these plots were scaled by setting 2·65 as the maximum response (= 1·0 in Fig. 8). The K_D was 130 μM and the Hill coefficient was 1.

Calcium responses were not suppressed during two successive carbamylcholine applications. Figure 7*B* demonstrates Ca^{2+} responses induced by two 5 mM-carbamylcholine puff applications separated by 140 s; these two responses were almost identical in amplitude. The amplitude of the second response was $108 \pm 6\%$ ($n = 20$) of the first one. The dose dependence of the Ca^{2+} response was measured by puff-applying carbamylcholine at various concentrations (Fig. 7*B*). Responses to 100

μM-carbamylcholine measured in the same hair cell were similarly used as a reference. The dose–response relationship plotted in Fig. 8 (●) was scaled by setting 4·5 as 1·0. The K_D was 340 μM and the Hill coefficient was 1. Figure 8 also shows the ACh dose–response relationship to be just between those for muscarine and carbamylcholine. The differences in potencies among these three agonists were small, but the sequence was in the order of muscarine > ACh > carbamylcholine.

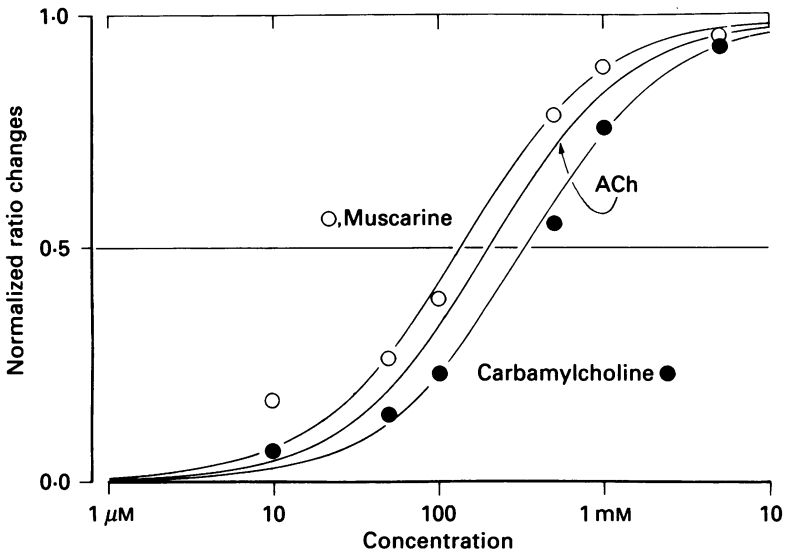

Fig. 8. Dose–response relationships measured by muscarine and carbamylcholine stimulation. Relative amplitudes of Ca^{2+} responses measured in experiments in Fig. 7 are plotted after normalization. ○, relative response amplitudes to 100 μM-muscarine (10 μM, 0·482±0·03, $n = 7$; 50 μM, 0·667±0·07, $n = 4$; 500 μM, 2·07±0·19, $n = 21$; 1 mM, 2·39±0·16, $n = 19$; 5 mM, 2·51±0·14, $n = 25$; the maximum amplitude was assumed to be 2·65); ●, relative response amplitudes to 100 μM-carbamylcholine (10 μM, 0·298±0·11, $n = 26$; 50 μM, 0·670±0·03, $n = 22$; 500 μM, 2·39±0·18, $n = 19$; 1 mM, 3·37±0·22, $n = 19$; 5 mM, 4·18±0·35, $n = 16$; the maximum amplitude was assumed to be 4·5). Two continuous lines were drawn according to the Michaelis–Menten theory with a K_D of 130 μM for muscarine, and 340 μM for carbamylcholine. The Hill coefficient was 1 for both curves. The dose–response relationship for ACh responses is included.

ATP induces intracellular Ca^{2+} responses

Co-existence of adenosine triphosphate (ATP) with ACh has been demonstrated in synaptic vesicles and in nerve terminals (reviewed by Burnstock, 1981). These two agents are likely to be released simultaneously when presynaptic nerve endings are excited. ATP causes contraction of the urinary bladder while its dominant effect seems to be hyperpolarization of the smooth muscle by activation of Ca^{2+}-activated K^+ conductances. ATP effects on the intracellular Ca^{2+} concentration were studied in the hair cell.

When ATP was puff-applied to the hair cell, it generated Ca^{2+} responses. The time course of the ATP-induced Ca^{2+} response was different from that induced by the muscarinic agent (Fig. 9*A*). Muscarine was puff-applied to hair cells at 50 s in Fig. 9*A*. It generated a long-lasting Ca^{2+} response with a slow rising phase. A puff of ATP

(100 μM) generated a much larger Ca^{2+} response (at 230 s in Fig. 9*A*) and a rapid rising phase was followed by a slow increase of intracellular Ca^{2+}. The rapid rising phase is much clear in Fig. 10*A* (at about 40 s in trace 4 particularly). Since the time resolution of the present method is limited to 3 s, the exact timing to the ATP

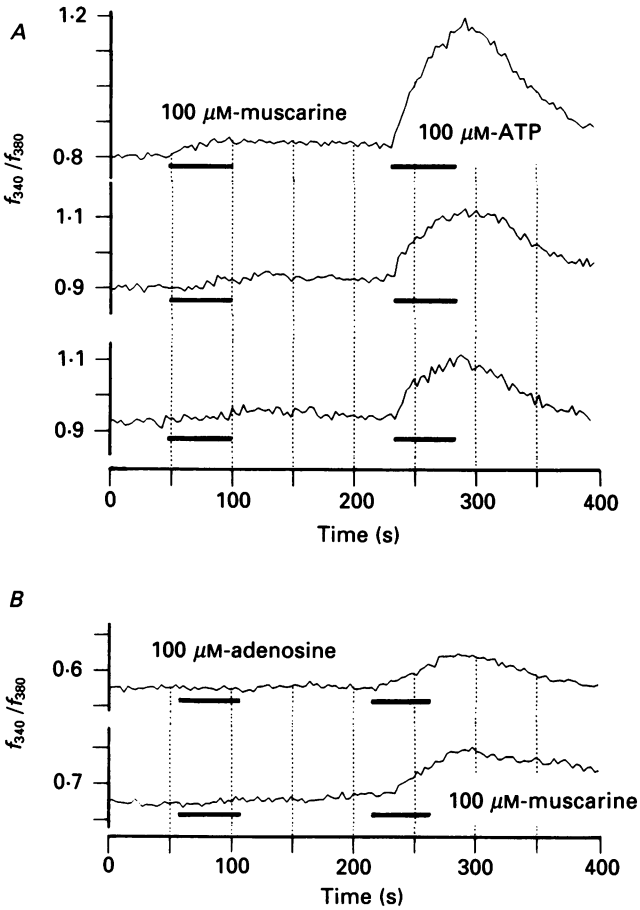

Fig. 9. ATP induces Ca^{2+} responses in hair cells. *A*, 100 μM-ATP-induced Ca^{2+} responses in hair cells. The response had a more rapid rising phase and larger amplitude than that induced by 100 μM-muscarine. Puff applications are indicated by bars in each trace. *B*, adenosine at the concentration of 100 μM did not induce Ca^{2+} responses in hair cells where clear muscarine-induced Ca^{2+} responses were generated.

application and the exact time course of this rapid phase cannot be detected. However, the rapid phase appears to be completed within one measurement cycle (3 s). When ATP puff was applied before muscarine, muscarine did not generate Ca^{2+} response in all ten hair cells studied. From ATP-induced Ca^{2+} responses at 1, 10 and 100 μM-ATP, the K_D may be about 10–20 μM.

When adenosine (100 μM) was puff-applied, no Ca^{2+} response was generated (Fig. 9*B*); a muscarine-induced Ca^{2+} response was observed normally from the same hair cell. Ca^{2+} responses induced by ATP in the hair cell might be mediated by a type P_2

purinergic receptor (Burnstock, 1981). We have not studied the effect of specific blockers, but blockers of type P_2 purinergic receptor, quinidine and apamin, are also known to be blockers of Ca^{2+}-activated K^+ conductance (Cook & Haylett, 1985).

ATP activates Ca^{2+} responses in two phases

Since ATP activates Ca^{2+} responses so rapidly, the basic mechanisms may be different from those of the muscarinic receptor, which releases Ca^{2+} ions from an intracellular reservoir. An alternative to the intracellular release mechanism would be an influx of Ca^{2+} ions. ATP was puff-applied in Ca^{2+}-free medium in Fig. 10. The extracellular medium was replaced by the fast microperfusion method with a Ca^{2+}-free medium at 150 s; this was reflected in changes of fluorescence excited at 340 and 380 nm wavelengths, as demonstrated in Fig. 10*B*. These fluorescence changes indicate a decrease of the intracellular Ca^{2+} concentration. When such a decrease of intracellular Ca^{2+} concentration became stable, ATP (100 μM) in the same Ca^{2+}-free medium was puff-applied at 210 s. It generated a slow rise in the intracellular Ca^{2+} concentration. Note that the control ATP puff (at 40 s) in normal saline generated both rapid and sustained slow rising phases, while the puff of ATP with 2·5 mM-Ca^{2+} generated only a rapid Ca^{2+} response at 360 s. This experiment suggests that the first rapid phase is due to an influx of Ca^{2+} ions and the second slow phase is a release of intracellular Ca^{2+} ions. Since ATP causes a substantial release of Ca^{2+} ions from the intracellular reservoir the reservoir might be easily depleted when extracellular Ca^{2+} source is eliminated. Therefore, the second puff of ATP with 2·5 mM-Ca^{2+} made in the Ca^{2+}-free bathing medium might have generated only the Ca^{2+} influx component (fast response).

Electrical membrane responses generated by ACh and ATP

The rise of intracellular Ca^{2+} concentration has influences upon at least one component of ionic channels of the hair cell membrane. Since the hair cell membrane is equipped with Ca^{2+}-activated K^+ channels (Lewis & Hudspeth, 1983; Ohmori, 1984), the rise of intracellular Ca^{2+} concentration is likely to activate K^+ conductance and to hyperpolarize the membrane. As expected, a puff of 50 μM-ACh generated hyperpolarizing membrane potential responses from the resting level of -50 mV to -74 mV (Fig. 11*A*). The response was extremely long lasting (about 6 min), and fluctuated between about -55 and about -70 mV. The initial part of the voltage fluctuation is illustrated by expanding the time scale in Fig. 11*B*. The voltage fluctuation appeared again in the recovery phase of the ACh-induced hyperpolarization at about the same membrane potential (Fig. 11*A*). These voltage fluctuations probably reflect fluctuation of the outward current generated by opening and closing of the Ca^{2+}-activated K^+ channels.

When 500 μM-ATP was puff-applied to a voltage-clamped hair cell (holding potential, -50 mV), it generated an inward current almost instantaneously, and then a slow outward current (Fig. 11*C*). The outward current subsided slowly. This ATP-activated inward current is likely to be responsible for the rapid component of the ATP-activated Ca^{2+} response (Figs 9 and 10). The succeeding slow outward component is probably the Ca^{2+}-activated K^+ current, but it has not been studied in detail.

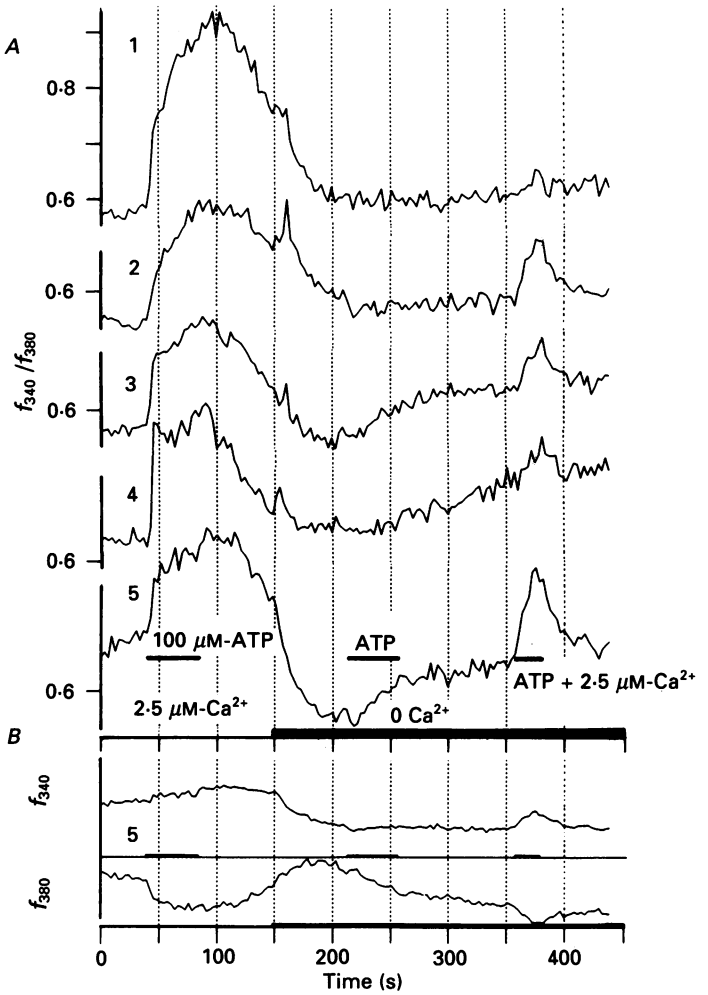

Fig. 10. ATP generates Ca^{2+} responses in Ca^{2+}-free extracellular medium. *A* and *B*, Ca^{2+}-free extracellular medium was microperfused during the period indicated by the bar on the abscissa. Since the flow of Ca^{2+}-free medium was so rapid it displaced cells and generated spike-like artifacts in the fluorescence ratio traces (at about 150 s in traces 2, 3 and 4 in *A*). The flow of Ca^{2+}-free medium reduced the 340 nm fluorescence and intensified the 380 nm fluorescence reciprocally, indicating a decrease of intracellular Ca^{2+} concentration (*B*). When this decrease of intracellular Ca^{2+} concentration was stabilized, 100 μM-ATP was puff-applied and a slow increase of intracellular Ca^{2+} concentration was induced in traces 2, 3 and 4 and 5 (*A*). When 100 μM-ATP with 2·5 mM-Ca^{2+} was puff-applied in Ca^{2+}-free bathing solution (at about 360 s), a rapid Ca^{2+} response was generated but the total time course of the Ca^{2+} response was much shorter than the control (starting at 40 s).

DISCUSSION

We have studied Ca^{2+} responses generated by cholinergic muscarinic agonists and ATP within dissociated chick hair cells. After puff application of muscarinic agonists, a rise of intracellular Ca^{2+} concentration was observed. Since the Ca^{2+} response could

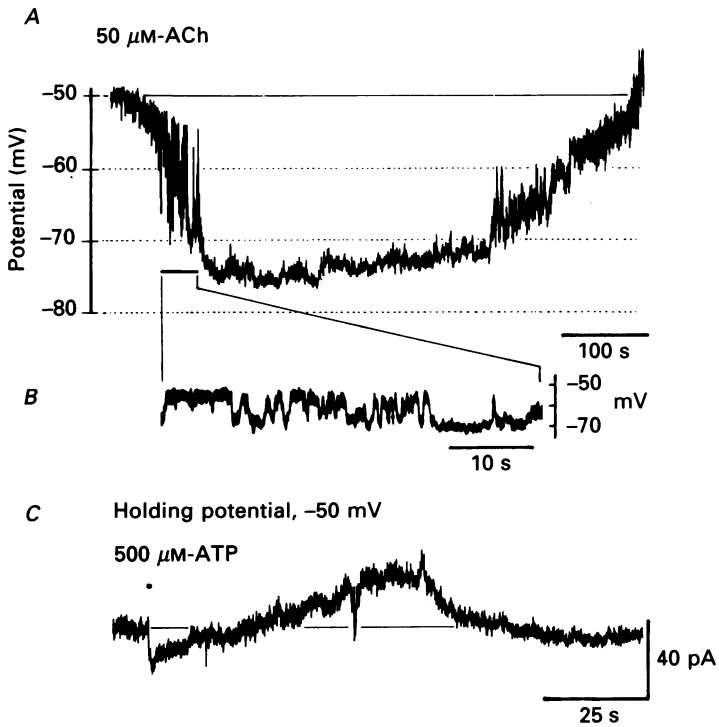

Fig. 11. Hair cell electrical responses generated by ACh and ATP puff application. Hair cells were whole-cell current or voltage clamped with a KCl, EGTA-based intracellular medium and normal extracellular medium. Application of a puff of ACh and ATP is indicated by a bar. *A*, 50 μM-ACh was puff-applied for 1 s to a current-clamped hair cell and a hyperpolarizing potential response was generated. *B*, an initial phase of the ACh-induced membrane hyperpolarization was illustrated on an expanded time scale. A corresponding time period is marked by a bar in Fig. 11*A*. *C*, a puff of 500 μM-ATP for 1 s generated an inward and outward current in a voltage-clamped hair cell. The outward current subsided slowly after reaching the maximum. The membrane potential was held at −50 mV.

be observed in Ca^{2+}-free medium, muscarinic agonists probably release the intracellularly stored Ca^{2+} ions. ATP appears to generate intracellular Ca^{2+} increases by two processes; one by influx of extracellular Ca^{2+} ions and the other by release of intracellular Ca^{2+} ions.

In these experiments, hair cells were loaded with the membrane-permeable ester form of Fura-2 AM and fluorescence intensities were measured by exciting at two wavelengths, 340 and 380 nm. The fluorescence signal has been complicated in rat peritoneal mast cells loaded with the ester form of the dye because a significant fraction of the dye accumulated into secretory granules within the cell (Almers & Neher, 1985). Both the Ca^{2+}-insensitive wavelength (350–360 nm) and the Ca^{2+}-sensitive wavelength (390 nm) fluorescence were decreased by degranulation in the mast cell, and apparently only a small intracellular Ca^{2+} response was indicated.

However, when the mast cell was loaded with the membrane-impermeable form of Fura-2, only the 390 nm fluorescence was decreased by the degranulation and a rapid rise of the intracellular Ca^{2+} concentration was indicated. These complications were not observed in the chick hair cell: (1) the Ca^{2+} responses induced by ACh and its analogues and by ATP in the chick hair cell have always generated reciprocal changes of the fluorescence intensities excited at 340 and 380 nm wavelengths (Figs 1*B* and 10*B*); (2) fluorescence intensities were measured from multiple points along the cell body in some solitary hair cells, but regional differences were not observed at all in the time course of the fluorescence changes (Fig. 1); (3) although ATP generated Ca^{2+} responses in two kinetics, the slow phase of the Ca^{2+} response was due to an intracellular release mechanism, while the rapid phase was due to an influx of Ca^{2+} ions from the extracellular space (Fig. 10*A*). We may not have eliminated the possibilities of specialized intracellular compartments by these observations alone, but we don't have any good reasons to propose their presence either. We therefore consider it likely that the Fura-2 fluorescence monitored in these experiments reflected the cytosolic Ca^{2+} concentration rather than the Ca^{2+} concentration of a specialized compartment within a hair cell.

Muscarinic receptors of other preparations may have a slightly higher sensitivity to agonists than that studied in this paper: in the heart, potassium conductance is activated in atrial trabeculae or in the isolated myocardial cells at 1 μM or at lower ACh concentrations (Giles & Noble, 1976; DiFrancesco, Ducouret & Robinson, 1989). In exocrine glands, 0·5–2 μM-carbamylcholine or 10 μM-ACh activates K^+ conductance (Trautmann & Marty, 1984; Gallacher & Morris, 1987). In the fibroblast A9 L cell line, 10–50 μM-ACh induced hyperpolarization of the membrane (Jones, Barker, Bonner, Buckley & Brann, 1988). Superior cervical ganglion neurones of rabbit demonstrated a slow depolarization of the membrane by bath application of 10 μM to 1 mM-ACh (Dun, Kaibara & Karczmar, 1978). In the lateral line organ, Russell (1971) has inhibited afferent nerve activity with 1 μM-ACh applied together with 5 μM-physostigmine. A carbamylcholine concentration of 50–500 μM was required for clear suppression of afferent nerve activity there. In turtle hair cells, 100 μM-ACh was applied together with 20 μM-neostigmine in order to induce hyperpolarization of the membrane (Art *et al.* 1984). Therefore, the ACh concentration (100 μM) we have routinely used to activate intracellular Ca^{2+} responses was relatively high but within the range used by others to activate muscarinic responses in some preparations and mimic efferent stimulation responses in hair cells.

The cholinergic muscarinic receptor we studied in this paper might have a similar sensitivity to those receptors investigated in neurones or in the exocrine glandular cells. We have observed a clear Ca^{2+} response activated by 1 μM-ACh (Fig. 4*B*). However, the fluorescence measurements we have adopted might be less sensitive than the electrophysiological detection technique, since the Ca^{2+} response detected here could have been an average of a certain area. Any local Ca^{2+} responses would be diluted by intracellular Ca^{2+} diffusion processes before they could be detected as a fluorescence signal here, while a membrane electrical response might have been activated directly by a local phenomenon *per se*.

The time course of the Ca^{2+} response, and the membrane hyperpolarization induced by an ACh puff, were much longer than the inhibition induced by efferent

nerve stimulation in hair cells or in afferent nerves (several hundred milliseconds; reviewed by Klinke & Galley, 1974). This slow rise and prolonged change of the intracellular Ca^{2+} response cannot be explained by the simple diffusion and wash-out time of the agonist: (1) ATP generated a rapid rise of the intracellular Ca^{2+} concentration while muscarine induced only a slow Ca^{2+} response in the same cell (Fig. 9*A*); (2) the perfusion rate of the bathing medium was fast and the volume of 6–10 times the recording chamber was exchanged every minute (see Methods); (3) the agonist clearance was rapid and was less than 3 s (see Methods). On the other hand, we have applied relatively high concentration of agonists in this paper as discussed already and might have prolonged the responses. Under physiological conditions, ACh would be removed promptly from the receptor area by ACh esterase hydrolysis (Churchill *et al.* 1956; Iurato *et al.* 1971). Concentrated application of ACh as a packet and prompt removal of agonist might shorten the total response period.

In some afferent fibres, efferent stimulation has induced facilitation of the spike activities. This would probably be accompanied by an increase in the membrane impedance (Highstein & Baker, 1985). Some primary afferents might have another type of muscarinic receptor which reduce the K^+ conductance and increases the membrane impedance (Krnjević, 1974).

The cholinergic muscarinic receptor is a likely candidate for the efferent innervation mechanism of the hair cell (reviewed by Klinke & Galley, 1974). Synthesis (presence of choline acetyl transferase), break-down (presence of ACh esterase), increase of ACh content in the perilymph after efferent stimulation, mimetic effects of exogenously applied ACh to the efferent nerve stimulation, and inhibitory effects of cholinergic antagonists to efferent stimulation have been demonstrated (reviewed by Guth *et al.* 1976). This paper has further demonstrated an ACh-induced intracellular Ca^{2+} mobilization as a basic mechanism for the hyperpolarization of the hair cell membrane. This cholinergic muscarinic receptor mechanism is likely to mediate the inhibitory efferent innervation of the olivocochlear bundle to the hair cell. CGRP may exist in this efferent synapse as a neurotrophic factor. If ATP co-exists with ACh in the efferent synapse, its depolarizing effects might be contributing the depolarizing efferent responses detected in turtle hair cells (Art *et al.* 1984).

The authors thank Dr M. E. Barish for careful reading of the manuscript, and Dr J. F. Ashmore for comments on the manuscript. We are grateful to Mr Y. Itoh and Ms M. Yoshitomo for their assistance in electronics, setting up of a computer, and for graphic displaying and photographs. This paper was supported by a grant-in-aid for developmental scientific research (62870005) to H.O.

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